Immuno-affinity purification of mouse E2F8 protein complexes

A Senior Honors Thesis

Presented in partial fulfillment of the requirements for graduation with distinction in Molecular Genetics in the College of Biological Sciences of The Ohio State University

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May 2006

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Abstract

The E2F family of genes has been extensively studied in the hope of gaining insight into the role of these DNA binding proteins. The general function of all E2F proteins is to bind to DNA and regulate the expression of a multitude of genes responsible for cellular proliferation. Given the central role the E2F family plays in the development and progression of cancerous cells, it is imperative to understand the pathways they are involved in and just how they are regulated.

Shortly after the complete human and mouse genomes were finished, the final E2F family member was discovered: E2F8. The characteristics of this gene have only begun to be satisfactorily identified. It is known that the protein possesses two distinct DNA binding sequences, quite similar to its closely related family member E2F7. Like E2F7, over-expressed E2F8 has shown an ability to inhibit cellular proliferation, which suggests that both proteins act as tumor suppressors.

A great majority of proteins do not function alone; they must interact with other proteins in order to fulfill their determined function in the overall metabolic scheme of the cell. If one is to understand the nature of a protein and the role it carries out in cellular pathways, the protein complexes in which the protein participates must be identified.

The specific aims of this project are to first express a tagged version of E2F8 in HeLa cells, and then to use that tagged protein as bait to isolate binding partners. To this end, a double-tagged version of E2F8 is subcloned into a retroviral vector that couples the construct with the ILR2-alpha receptor sequence via an IRES. The vector is transfected into Phoenix packaging cells which then produce human specific, infectious virions. This virus is used to transduce HeLa cells and consequently over-express E2F8 ectopically. Expression of E2F8 also constitutes IR2-alpha expression and this membrane receptor is used to enrich the E2F8

expressing cells through magnetic bead affinity. Enrichment of E2F8 is confirmed by immunoblot analysis.

The over-expressing cell-lines are grown in large, 8 liter suspension culture batches in order to produce sufficient levels of protein for isolation and eventual mass-spectrometry analysis. The large batches are harvested and lysed in such a way as to isolate cytoplasmic content separately from nuclear. This allows for a concentration of nuclear proteins, among which exist our complexes of interest. The nuclear lysates are then allowed to bind to resin conjugated to antibody raised against one of the tagged sequences, in this case, FLAG. The bound complexes are washed and then eluted. This eluent is then further filtered by repeating the purification using another antibody bound matrix, in this case, HA (hemagglutinin peptide sequence). The final elution is screened for E2F8 and binding partners via immunoblotting and silver staining, and once the identities are confirmed, they are sent out for mass-spectrometric analysis. The relationship between E2F8 and its binding partners will be further pursued once this data is obtained.

Introduction

The E2F transcription factor family plays a large role in the regulation of the cell-cycle. The general function of all E2F proteins is to bind to DNA in a sequence specific manner and regulate the expression of a multitude of genes responsible for cellular proliferation. If particular E2F pathways are somehow disrupted, unregulated cellular replication will inevitably ensue, and a tumor will form (21). It has been shown that a disruption in the retinoblastoma pathway or an alteration in Cdk activity that leads to the deregulation of E2F is a property of virtually all cases of human cancer (14, 18). Given the central role the E2F family plays in the development and

progression of cancerous cells, it is imperative to understand the pathways they are involved in and just how they are regulated.

The E2F family is generally divided into at least two classifications: transcriptional activators or transcriptional repressors. The E2Fs that are considered transcriptional activators include E2F1, E2F2, and E2F3a. Their peak expression levels are found in the late G1 phase of the cell-cycle where they proceed to associate with E2F-regulated promoter regions during the G1-S transition. These E2F target genes are transcribed and facilitate movement into S phase. It has been demonstrated that over-expression of E2F is sufficient for S phase entry (15, 16) and that cells that have E2F1-3 inactivated are defective in G1-S transition (17), indicating that their role is vital in maintaining proficient and regulated cellular proliferation. In contrast to the activator E2Fs, the repressors E2F3b, E2F4, and E2F5 are all expressed throughout the cell-cycle, yet seem to function mainly in quiescent cells. These proteins associate with E2F-target promoters throughout G0 (1-3). In addition, E2F6 acts as a repressor by target promoter association facilitated by protein recruitment of polycomb group proteins or complex formation with Mga and Max proteins (4, 5).

E2Fs 1-5 associate with the tumor suppressor protein Rb, which is necessary for controlled cellular division, differentiation, and apoptosis (13-15). The E2F activator proteins are bound by Rb. In the Rb-bound state they remain inactive until cyclin dependent kinases phosphorylate Rb and cause a conformational change in the protein which releases the E2F factor. These released factors up-regulate E2F target genes which induces G1-S transition as described earlier. Rb also associates with some of the repressor E2Fs. In so doing, Rb recruits chromatin-remodeling complexes and DNA and histone-modifying enzymes such as DNA methyltransferases and HDACs, respectively (6-12). These complexes are also cell-cycle

specific, in that phosphorylation of Rb causes the repressive complexes to dissociate. E2F6, E2F7, and E2F8 do not contain Rb binding sequences. However, E2F8 may be the only E2F not regulated by the Rb pocket proteins (5, 19, 20).

E2Fs 1-6 are able to bind DNA in a sequence specific manner via their highly conserved DNA-binding and dimerization domains. In order to bind DNA efficiently, these E2Fs must heterodimerize with DP1 or DP2 (13-15). In contrast, E2F7 and E2F8 are unique in that they do not contain a dimerization domain but instead have a duplicated DNA-binding domain (19, 20). This allows these two proteins to bind DNA independent of DP1 and DP2. Furthermore, there is evidence that E2F7 and E2F8 both homodimerize, and can actually form heterodimers with one another (19, 20, Li unpublished data). This evidence suggests that E2F7 and E2F8 could have overlapping or even synergistic functions in the cell.

Unlike E2F7, E2F8 does not code for transactivating regions or pocket protein binding domains. E2F8 consists of 890 amino acids with an approximate weight of ~95 Kd. The mouse tissues in which this protein is most highly expressed are the liver, skin, thymus, and testis, in contrast to the brain, muscle, and stomach, where E2F8 expression has not been detected or is too low for experimental consideration (19). E2F8 exhibits a similar expression pattern as the activator E2Fs: they are all up-regulated at the G1/S transition (18). However, E2F8 is not an activating protein, in fact, ectopic expression of E2F8 in MEFs (19) or human TIG3 fibroblasts (18) causes a marked inhibition of cellular proliferation and down-regulation of E2F target genes. In addition, cells over-expressing E2F8 constructs bearing point-mutations in either of their DNA-binding domains do not exhibit either of these phenotypes and divide at a similar rate to wild-type cells (18, 19). Therefore it is the ability of E2F8 to bind DNA in a sequence specific manner that allows it to negatively affect cellular proliferation. Also, the

expression pattern of E2F8 suggests that it acts in a competitive manner with activator E2Fs for target promoters (18).

This experimental evidence indicates that E2F8 is a tumor suppressor candidate. In order to elucidate the full capabilities of E2F8, we must know what biochemical interactions it undergoes in the cell. It is known that E2F8 binds to DNA (18) and can form dimers (19). Further studies are needed to discover what other proteins E2F8 interacts with in the nucleus. Indeed, a great majority of proteins do not function alone; they must interact with other proteins in order to fulfill their determined function in the overall metabolic scheme of the cell. In so doing, proteins form complexes (Fig. 1). That is, two or more proteins bind together to form a quaternary structure that affords the ability to perform cellular functions, the likes of which the complexes' components alone are not capable of, or cannot carry out with the same efficiency. If we are to fully understand the potential of E2F8 and the role it carries out in cellular pathways, the protein complexes in which it participates must be identified (4).

The aim of this experiment is to isolate E2F8 protein complexes from Hela cells and to identify novel binding partners via mass-spectrometric analysis. In order to do so, E2F8 is tagged with two sequences specific for designated antibodies, in this case FLAG and HA. This construct is subcloned into a retroviral vector that also produces an ILR2-alpha receptor via an internal ribosomal entry site. This vector is packaged and then used to transduce HeLa cells. Cells efficiently producing E2F8 are selected using magnetic beads coupled to antigen specific for the ILR2-alpha surface receptor. Enriched cell lines are then grown up in large suspension culture batches and lysed. The nuclear content is separated and the tags on the N-terminus of the E2F8 construct are used to purify and separate the complexes from other cellular matter (Fig. 2). The components of these complexes are determined by mass-spectrometric analysis. Here we

identify two novel binding partners: HDAC1 and SIN3A. This data was obtained via the described purification method followed by immunoblot analysis. Results from the mass-spectrometry labs ought to identify further E2F8 binding partners.

Materials and Methods

Retroviral Vector Subcloning:

Standard subcloning protocols were employed to place the 2583 bp E2F8 sequence in frame with Flag and HA tags and an IRES and IL2Ralpha sequence, including Qiagen mini and maxi prep kits, PCR amplified segments and oligo sequences designed to include necessary restriction enzyme sites for proper placement of the construct in the pOZFHN retroviral vector. Further subcloning was needed to also produce several mutant constructs of E2F8 within the pOZFHN plasmid, one with point mutations of the conserved leucine 118 and glycine 119 in the first DNA binding domain (DBD-1), another with point mutations of the conserved leucine 266 and arginine 267 in the second DNA binding domain (DBD-2), and a third with point mutations in both DNA binding domains (DBD-1,2). Subcloning details are available upon request.

Amphitropic Phoenix Packaging Cell Line Transfection:

Amphitropic phoenix retrovirus packaging cells were thawed and split according to standard tissue culture protocol. $CaCl_2$ and HBS were thawed from -20ºC in a 37ºC water bath. Approximately 25 ug of plasmid DNA per p100 was added to 1 ml of $CaCl_2$ per p100. While bubbling 1 ml of HBS per p100 with a pipette, the $CaCl_2$ and DNA mixture was added drop wise to the HBS. The HBS, $CaCl_2$, and DNA mixture was immediately added drop wise to approximately 60% confluent p100 plates containing Phoenix cells in 15% FBS/DMEM. This

process was repeated for three plasmids: a control pOZFHN vector containing no construct, the pOZFHN vector containing the wild-type E2F8 sequence, and the pOZFHN vector containing a point mutation in the first DNA binding domain. All transfected plates were incubated at 37ºC incubator for approximately 20 hours, at which point the media was removed and the cells were washed twice with PBS. 6 ml of media was placed on the cells and they continued to grow in the incubator.

HeLa Cell Transduction:

HeLa cells were thawed and split according to standard tissue culture protocol. Virus was harvested from the transfected Phoenix cells by placing the virus containing media in 15 ml conicals with 3 ml of polybrene per p100. The conicals were spun at 3800 rpm for 10 minutes. Meanwhile, media was added back to the Phoenix cell plates and these were placed back in the incubator. HeLa plates with ~60% confluence were taken out of the incubator and the media was removed. The supernatant was removed from the conicals without disturbing the virus pellet. 10 ml of media was added to each tube and the pellets were re-suspended. The contents from each conical were added to a plate containing HeLa cells. The HeLa cells were incubated for 5 hours and then the virus was removed and the media was replaced. Infections were repeated every 12 hours for a total of three infections. Cells were harvested for immunoblot analysis and E2F8 expression verification.

Magnetic Affinity Enrichment:

Bead suspension was prepared by adding 5 ul per sample of anti-IL2Ralpha antibody-conjugated magnetic beads to 5% FBS/DMEM and vortexed. Infected HeLa cells were split to T175 flasks

and 1 ml of bead suspension was added to each. The flasks were incubated at 37ºC for 30 minutes with occasional shaking. Bead binding was observed with a microscope. The suspension was transferred to a T25. The remaining cells were transferred using a PBS wash followed by 2 ml trypsinization. Trypsin exposure was minimalized to prevent degradation of the surface marker and DMEM was added quickly to neutralize the solution after the cells were detached. This suspension was also added to the T25 flask. This procedure was repeated for all samples. The T25 flasks were attached with a rubber band to a magnetic plate and stood vertically. The plates were rocked gently to bring the cells within magnetic range. After 30 minutes, unbound cells were aspirated from the flasks along with the media. 50 ml of DMEM was added to each T25 to wash the cells. The wash was repeated twice more. The flasks were removed from the magnetic plates and 5 ml of DMEM/15% FBS was added to each flask. Bead bound cells were observed under the microscope. The cells were allowed to grow at 37ºC and the sorting procedure was repeated again up to four times. Enrichment of E2F8 expression was verified by immunoblot analysis.

Large Batch Suspension Culture Maintenance:

Sorted HeLa cells were grown in 5 p150s per sample, the cells were counted, spun down, and then transferred to 2 liter flasks. Media was changed from 15% FBS in DMEM to 10% Bovine Calf Serum (BCS) in Joklik medium. Cell density was adjusted to 2 x 10^5 cells/ml. The flasks were placed in a 37ºC water bath over stir plates and magnetic stir bars rotated the media at approximately 60 rpm. Cell density was monitored every 24 hours and adjusted back to 2 x 10^5 cells/ml by adding Joklik media and BCS. As the volume increased, the cells were transferred to

12 liter flasks where the procedure was continued until 8 liters of cells at a density of ~1x10^6 cells/ml was ready to harvest.

Large Batch Harvest and Nuclear Extraction:

The cells were harvested by centrifugation at 1000 g (~3000 rpm) for 10 minutes and suspended in 5 volumes of cold PBS. The cells were maintained at 0-4ºC throughout the harvest. The cells were spun down again at 1000 g for 10 minutes and suspended in five volumes of Buffer A (10 mM Hepes (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF). The cells were left on ice for 10 minutes and then collected again by centrifugation. The cells were suspended in 2 volumes of Buffer A and then homogenized by 10 strokes with a glass Dounce Homogenizer (B pestle). The cells were checked microscopically for cell membrane lysis. The nuclear membrane was intact at this point. The homogenate was then centrifuged for 10 minutes at 1000 g. The supernatant was decanted and the nuclear pellet was centrifuged for 20 minutes at 25000 g. The supernatant was discarded and the nuclear pellet was re-suspended in 2.5 ml per 10^9 cells of Buffer C (20 mM Hepes (pH 7.9), 25% glycerol, 0.42 M NaCl, 1.5 mM Mg Cl2, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT and 0.1% Tween). This equates to approximately 20 ml for each 8 liter preparation. The nuclear pellet was then homogenized by 20 strokes of a glass Dounce Homogenizer and lysis was verified microscopically. The homogenates were then rocked for 30 minutes. The samples were then centrifuged for 30 minutes at 25000 g, and the supernatants transferred to 15 ml conicals. At this point samples were either snap frozen in dry ice and stored in -80ºC, or were immediately used for immunopurification.

Immuno-affinity Purification:

Frozen samples were quickly thawed in a 37ºC water bath, but were placed in ice before they were completely thawed to prevent complex degradation (the samples were always kept at or below 4ºC). The lysates were then spun down at 50,000 g for 30 minutes. The supernatants were transferred to fresh tubes and the step was repeated. While the extracts were spinning, M2 anti-FLAG antibody-conjugated agarose beads were prepared. 400 ul of beads per 10 ml of sample was washed in BioRad Poly-Prep Columns with 10 volumes of 100 mM glycine-HCl (pH 2.5) to remove uncrosslinked antibody. The beads were then washed with 10 bead volumes of 0.2 M Tris-HCl (pH 8). Buffer C was then used to wash the beads again (10 bead volumes). Then the column tip was capped and 1 bead volume of Buffer C was added to re-suspend the beads to a 50% slurry. The beads were then added to the supernatants and rotated overnight. Between 9 and 12 hours later, a column for each sample was setup over a 50 ml conical. The samples were then added to their respective column. The samples were allowed to drain by gravity flow. The flow through was then quick frozen and saved in -80ºC. The columns were then placed over glass flasks and the samples were washed by filling the columns to the top with Buffer C. The washing step was repeated twice more. The columns were then setup over a 15 ml conical and spun at 1200 g for 1 minute to remove excess liquid. The column tips were then capped and 400 ul of FLAG elution buffer (150 ug/ml of FLAG peptide in Buffer C) was added to each column. The columns were left in 4ºC for 1 hour. The columns were then placed over 15 ml conicals and spun at 1200 g for 1 minute. The samples were then transferred to 1.5 ml eppendorf tubes. About 50 ul of packed anti-HA 12CA5 antibody-conjugated beads were prepared per sample. The beads were washed in 1.5 eppendorfs with glycine-HCl (pH 2.5) followed by a wash with 200 mM Tris-HCl (pH 8.0). The beads were then re-suspended in Buffer C to a 50% slurry. The beads were added to the FLAG eluents and rotated for 30 minutes

in the cold room. The samples were then spun down at 14,000 rpm for <1 minute and the supernatant was snap frozen and saved in -80ºC. The beads were then washed three times with 2x the packed bead volume (100 ul) of Buffer C. The same volume (100 ul) of elution buffer (150ug/ml HA peptide in Buffer C) was added to the beads. The samples were incubated at room temperate for 30 minutes. The samples were then spun down and the supernatant was transferred to a fresh eppendorf and preserved with 5x loading dye. The samples were then stored at -80ºC and thawed for Western Blot analysis and Silver Stain analysis as needed.

Western Blot Analysis:

The immunopurified samples were separated in SDS polyacrylamide gels and transferred to polyvinylidene fluoride membranes. The membranes were rocked in blocking buffer (10% skim milk in Tris-buffered saline-Tween) for one hour at room temperature. The primary antibody was then added to 1% skim milk in Tris-buffered saline-Tween at a concentration specific to the tendencies of the particular antibody and rocked at 4ºC overnight (specific antibody conditions will be made available upon request). The primary antibody was then bound to horseradish-peroxidase-conjugated secondary antibody at a 1:5000 dilution in 2% skim milk in Tris-buffered saline-Tween at room temperature for 90 minutes. The secondary antibody was then detected by ECL reagent as described by the manufacturer (Amersham).

Silver Stain Analysis:

The immunopurified samples were separated in SDS polyacrylamide gels and then the gels were fixed overnight in 50% methanol, 12% acetic acid and 0.05% formaldehyde. They were then washed 3 times for 20 minutes each in 50% ethanol. The gels were pretreated for ~45 seconds

with 0.02% sodium thiosulfate. They were then washed 3 times for 30 seconds in 50% methanol. Immediately, the gels were impregnated with 0.2% silver nitrate, 0.075% formaldehyde impregnating solution for 20 minutes. After impregnation, the gels were rinsed with H_2O twice for 20 seconds each. They were then allowed to develop (6% sodium carbonate, 0.0004% sodium thiosulfate, 0.05% formaldehyde) between 30 seconds and 3 minutes depending on how quickly the reaction took place. As soon as the resolution was preferable, the reaction was neutralized with 50% methanol, 12% acetic acid (22).

Results

In order to verify the success of the subcloning, four plasmids, including three constructs, were transiently transfected into HeLa cells (tranfection protocol is identical to that found in Methods). The first plasmid was an empty pOZFHN control vector and of course there was no E2F8 expression. The second, third, and fourth plasmids were the wild-type E2F8 construct, the DBD-1 mutant, and the DBD-1,2 mutant all ligated into their respective pOZFHN vectors; expression was detected for all three via immunoblot analysis probed with anti-FLAG. These specific mutant constructs have been shown to be unable to bind DNA (19).

Once the functional expression of the constructs was confirmed, packaging of the vectors into Phoenix cells was carried out. HeLa cell infections followed and once again, successful expression was verified by probing a Western with anti-flag antibody.

HeLa cell expression was detectable but not concentrated. In order to enrich the cell cultures, high-expressing cells were selected for using anti-IL2Ralpha antibody-conjugated magnetic beads. Physical binding of the magnetic beads to the HeLa cells expressing the receptor were verified via microscopy (Fig. 3). The selection process was repeated a total of four

times, as this proved to be a sufficient number for maximal enrichment. Cells from each enrichment round were harvested for Western blots. These Western blots verified marked improvement in expression levels for each construct (Fig. 4).

Once maximal enrichment was achieved, large scale suspension cultures were initiated using the high-expressing cultures. At this point, a problem was encountered: the wild-type E2F8 over-expressing cells, despite the enrichment process, lost expression during the large-scale growth period. Cells over-expressing E2F8 are greatly inhibited in their ability to proliferate (18, 19). Given this fact, it seems as though the low-expressing cells outgrew the high-expressing cells over time, thus diluting E2F8 expression. This reduction in the presence of E2F8 made the large preps from this construct ultimately useless, as a great deal of protein is needed for detection purposes. As a consequence of this, an alternate course was sought. Given that the mutant constructs maintain the ability to homodimerize (19), it was decided that the DBD-1 mutant would be used. An E2F8 protein with mutations in one or both of its DNA binding domains prevents its ability to successfully bind target promoters. With this ability hindered, the mutant E2F8 cannot affect cellular division and thus cannot inhibit the growth of high-expressing cells. Therefore the DBD-1 mutant was able to be grown up in large, 8 liter suspension cultures in stead of the wild-type cell line.

Two 8 liter cultures were grown up in parallel: the DBD-1 mutant cell line and a cell line containing the empty vector control. These cultures were harvested simultaneously. The cells were lysed in such a way as to separate the cytoplasmic content from the nuclear content. As evidenced by the presence of a nuclear localization signal, data from immunofluorescent staining (19), and given that E2F8 is a transcription factor, it is known that E2F8 resides in the nucleus.

This method of harvesting allowed for further concentration of the E2F8 level in the lysate (Fig. 5).

Nuclear lysates from the DBD-1 mutant and control vector were snap-frozen and stored at -80ºC. These samples were quick thawed in 37ºC and bound to M2 anti-FLAG antibody-conjugated agarose beads. The columns were washed and the bound products were eluted. Samples from the flow through, wash, beads, and eluent were all saved for Western blot analysis (Fig. 6). After several FLAG purification rounds, standard conditions were arrived at for optimized binding and elution. Following the FLAG elution, the eluents were then to be bound to anti-HA 12CA5 antibody-conjugated beads. Unfortunately, the HA purification step proved much more fickle, which made the standardization difficult. The challenges were to ensure that E2F8 bound efficiently to the antibody, as the majority of E2F8 tended to remain in solution and consequently was extracted with the flow through, and to ensure that the complexes remained intact throughout the HA purification. Several methods remedied this: an increase in the number of HA beads, an increase in binding time, and a decrease in salt concentration from 420 mM to 150 mM (Fig. 8). The lower salt stringency likely allowed for more binding partners to remain during the washes. Other final changes that were made to the initial protocol include an increase in protease inhibitors to prevent complex degradation and HA column elution using Glycine pH 2.5 instead of HA peptide competition. These changes allowed for a clean and robust E2F8 sequential purification protocol as evident by silver stain analysis (Fig. 8).

Despite the setbacks, two E2F8 binding partners were identified, and these were used to verify successful complex purification via immunoblot analysis. The first identified binding partner and most likely candidate was histone deacetylase-1 (HDAC-1). HDACs are chromatin modifiers and consequent transcriptional regulators. They repress gene expression by

deacetylating the beta-amino acids of lysine located near the amino termini of core histone proteins. As HDAC proteins are known to aid repressive complexes, it was a likely candidate for E2F8 binding. In order to discover E2F8/HDAC binding, anti-HDAC-1 antibody was used to probe Western blots containing FLAG immunopurified samples (Fig. 7). SIN3A was detected the way HDAC-1 was, by using an anti-SIN3A antibody to probe a Western containing the immunopurified lysates. SIN3A is thought to be a scaffolding protein in repressive complexes. HDAC is known to directly bind to SIN3A (10), which suggests that E2F8 binds to SIN3A and SIN3A binds to HDAC. Although HDAC-1 was purified by using E2F8, it is likely that it does not directly interact with E2F8 but is actually held in the complex by SIN3A.

Discussion

Since the HA purification step had proven so difficult to replicate, mass-spectrometric analysis has not yet been pursued. However, there are now ample reserves of purified E2F8 complexes prepared for mass-spectrometric analysis. It is now simply a matter of identifying novel proteins linked specifically to E2F8. The newly identified binding partners will be verified via immunopurification and western blot analysis. Once the identities are confirmed, new paths and directions will be taken to elucidate the function of E2F8.

It is difficult to predict what E2F8 binding proteins will be identified in the near future. Likely candidates are proteins typically found in repressive complexes, such as chromatin modifiers like HDAC, other proteins contained in the SIN3 complex (SIN3B), and methyl transferases. Perhaps E2F8 interacts with polycomb group proteins like E2F6 does. It is known that E2F8 can dimerize with E2F7, and that E2F7 binds with CTBP (Li, Gordon, Leone, unpublished data). The mass-spectrometry results should prove very interesting.

Once results are available from mass-spectrometric analysis and novel binding partners are identified, it may be worthwhile to repeat the experiment again, only this time with a retroviral vector containing the wild-type E2F8 sequence expressed under a tetracycline-regulatable promoter. This system would allow for high E2F8 expressing cells to be selected for, enriched, and then collectively silenced in respect to E2F8 expression. The ability to turn off E2F8 production would allow for large-scale liquid culture preparation without losing concentrated expression. Before the large batch is harvested, the repression system would be removed and E2F8 expression would be activated. The benefit to this system is obvious: wild-type E2F8 could be used instead of the mutant construct. The reason this would be preferable is because some repressive complexes do not exist without an initial interaction. It is possible that the initial interaction for E2F8 is DNA binding; this DNA binding event may cause a tertiary rearrangement in the protein, thus opening binding pockets for other proteins to be recruited. This idea is pure speculation and is simply a suggestion for further experimental pursuit; it ought to be a consideration pending the mass-spectrometric results of this experiment.

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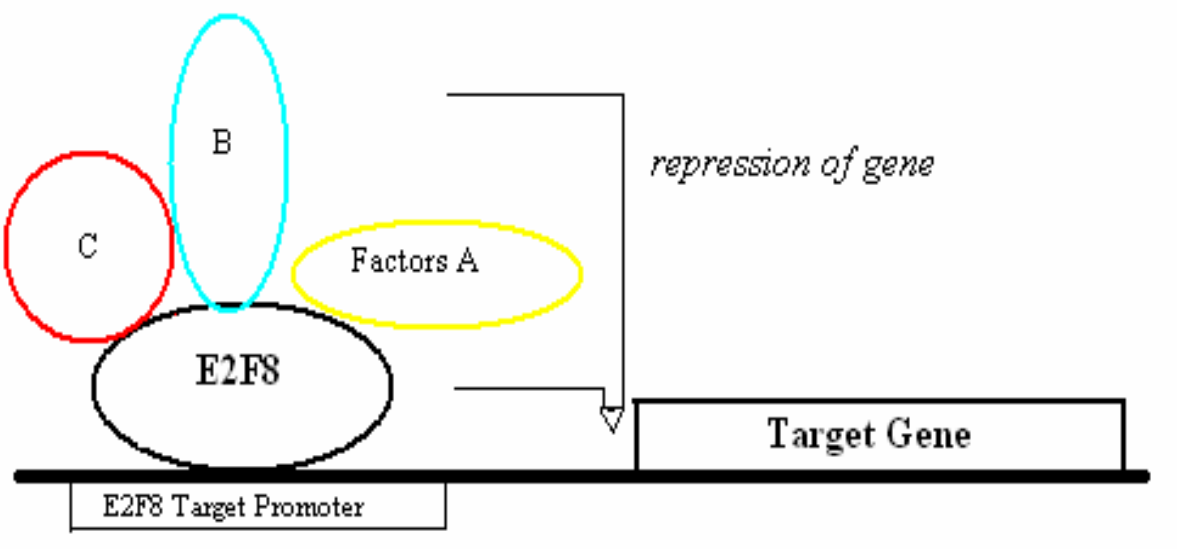

Figure 1. An illustration of an example of a protein complex recruited to a target promoter by the transcription factor E2F8. The complex forms to prevent expression of the downstream gene. Some or all of the proteins are needed to accomplish affective repression. If any of the co-factors are unable to bind, uncontrolled expression may occur, and in the case of E2F pathways, a tumor may eventually form.

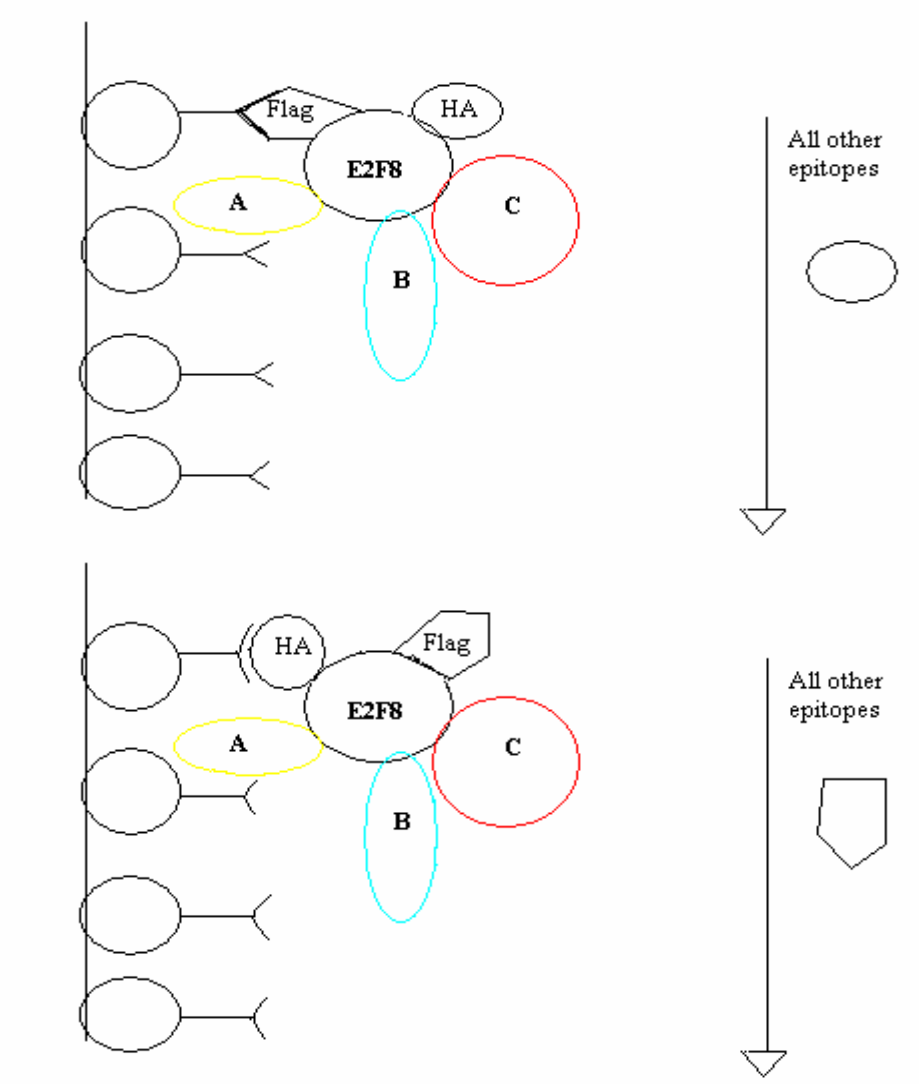

Figure 2. An illustration of the sequential immunopurification scheme. The FLAG epitope is able to bind in the first purification round (top), and consequently the E2F8 complex is also bound. All unbound material is washed away and discarded. Following elution, the sample is bound to HA beads (bottom) using the HA antigen. Once again, the complex is held in place while all unbound material is washed away. Any background material accumulated during the FLAG purification should be washed away during the HA purification.

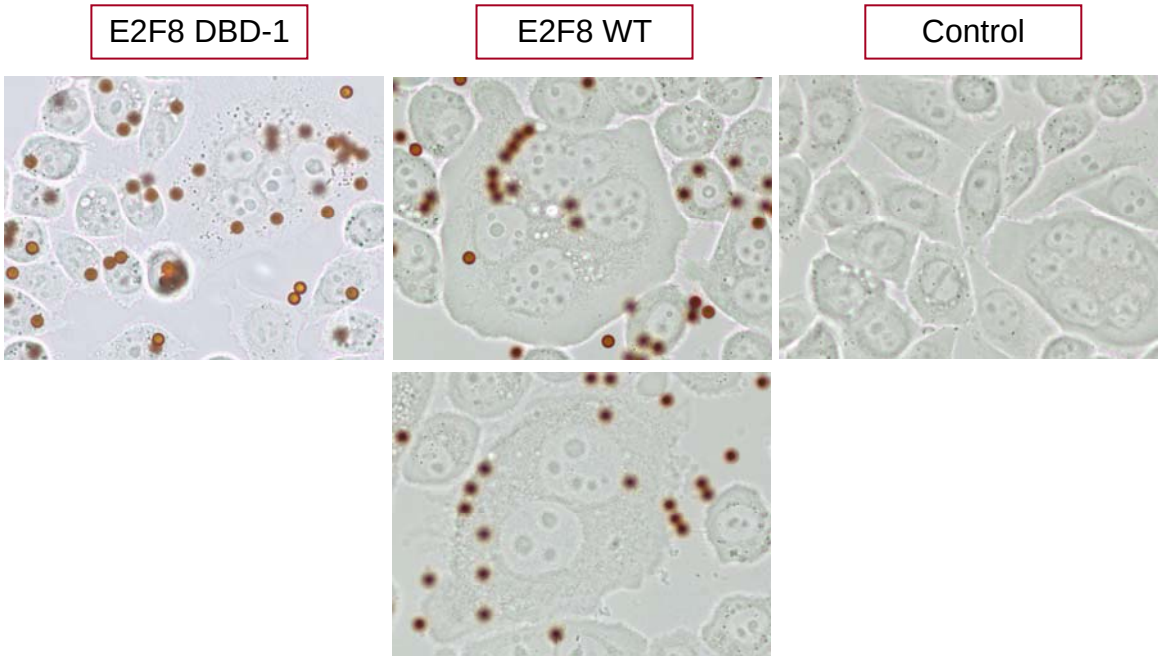

Figure 3. The upper left picture shows HeLa cells over-expressing E2F8 DBD-1 mutant protein. Consequently they are also expressing the IL2Ralpha receptor. The receptor is recognized by the anti-IL2Ralpha antibody-conjugated magnetic beads, which are bound to the surface of the cells displaying the protein. The orange circles are the beads. The center two pictures are also displaying the magnetic beads bound to HeLa cells, although these cells are over-expressing wild-type E2F8 instead of the DBD-1 mutant. The right most picture is the empty vector control; because the cells are not expressing a construct, the receptor protein is not expressed and no beads are bound.

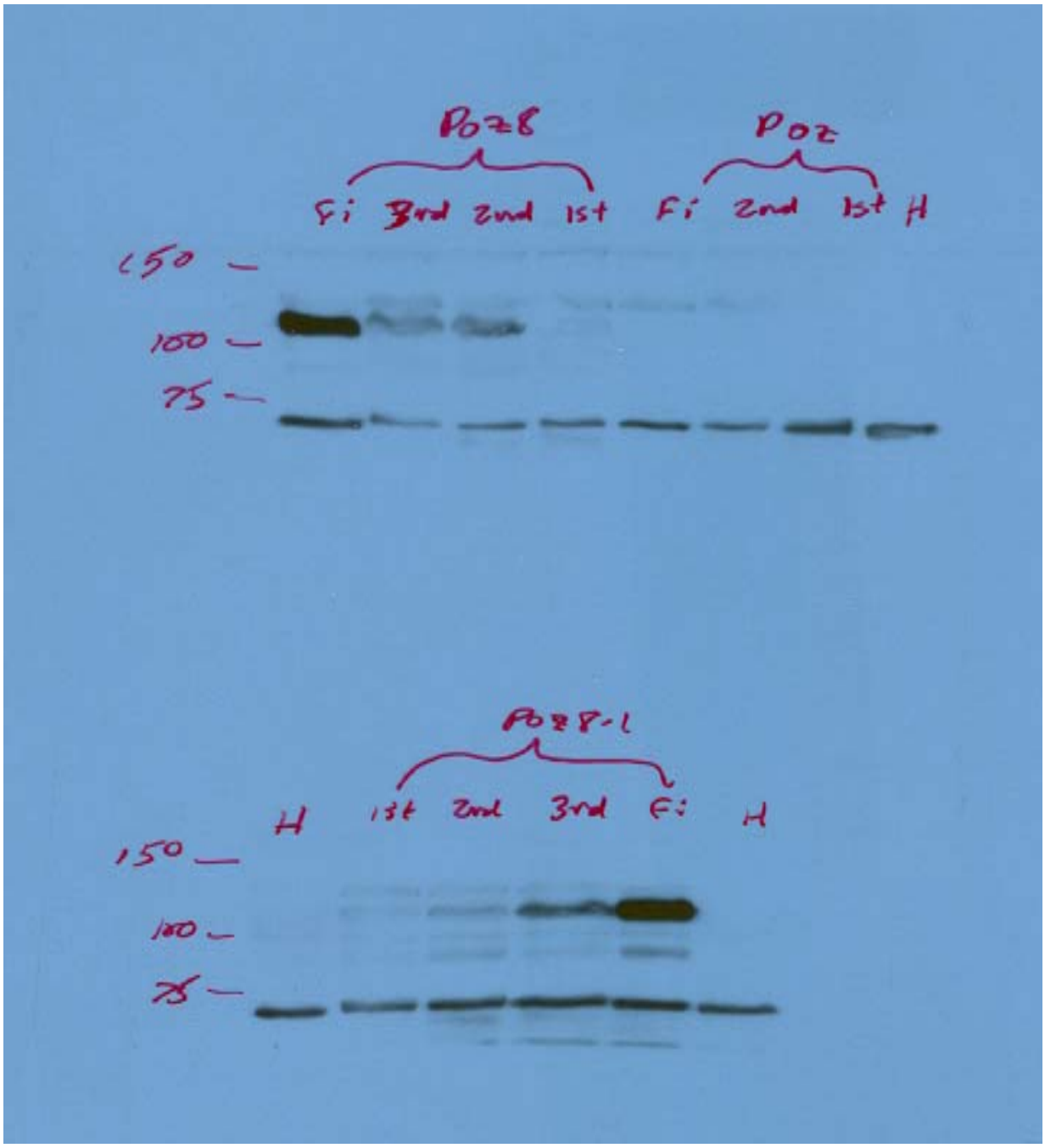

Figure 4. Western results for two gels containing samples saved from the magnetic bead sorting round. Both membranes were probed with anti-FLAG antibody. The top gel shows the progressive increase in E2F8 concentration for the wild-type cell line as compared to the empty vector control, where no change was seen. Untransfected HeLa cell lysate was also run as another control. The bottom gel shows the same enrichment process for the DBD-1 mutant cell line. HeLa lysate was run as a control.

Figure 5. A Western blot showing the sub-cellular localization of E2F8 and the successful nuclear harvest of HeLa cells over-expressing the construct. Untransfected HeLa cell lysate was run as a negative control. Sorted HeLa cells over-expressing E2F8 were harvested in a typical manner and run as a positive control. The nuclear extract contained E2F8 (~110 Kb) while E2F8 was undetectable in the cytoplasmic extract.

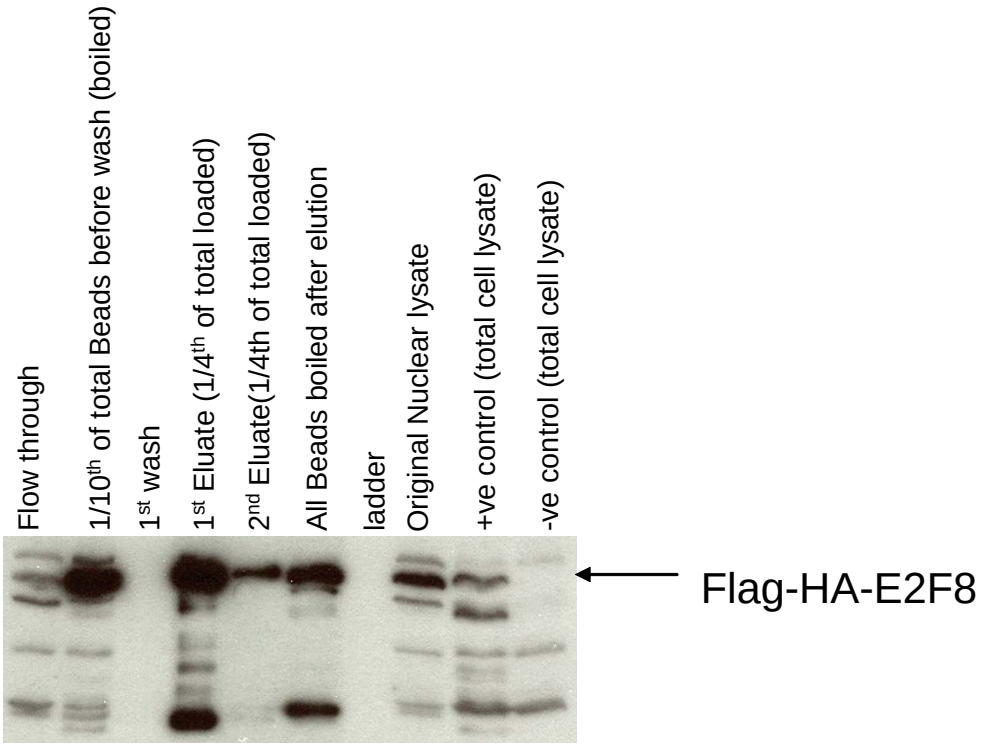

Figure 6. A Western blot showing the results of a FLAG purification. The left most lane is the flow through (unbound protein). To the right of that is the bound portion of protein at that point of the purification. To the right of that is what flowed through with the first wash. Next to that lane is the first eluent. To the right of the first eluent is the protein remaining bound after the first elution, which was eluted the second time. To the right of that are the proteins that did not elute but remained bound to the beads. After the ladder is the nuclear lysate (before IP) control lane, then the total cell lysate positive control, and finally, the total cell lysate negative control. Not all of the E2F8 protein eluted efficiently.

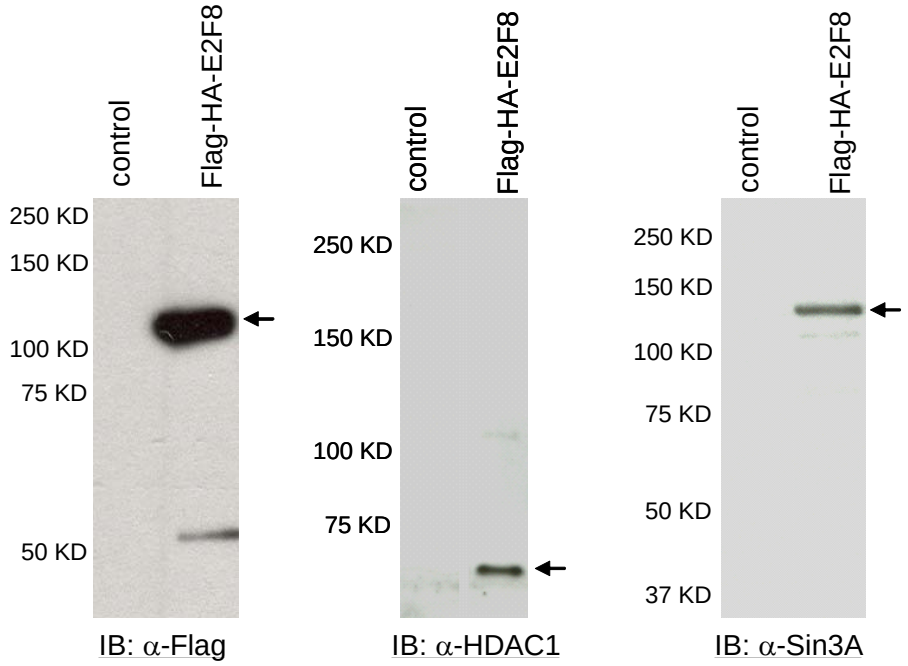

Figure 7. Three separate Western blots probed with three different antibodies. Each immunoblot contains FLAG immunopurified samples. The left picture shows E2F8 after FLAG immunopurification as compared to the empty vector control. The center picture shows the same samples probed with anti-HDAC-1 antibody. The right picture shows the same two samples as the other blots probed with anti-SIN3A antibody. This data confirms that the complexes were in fact intact and purified along with E2F8 through the FLAG purification.

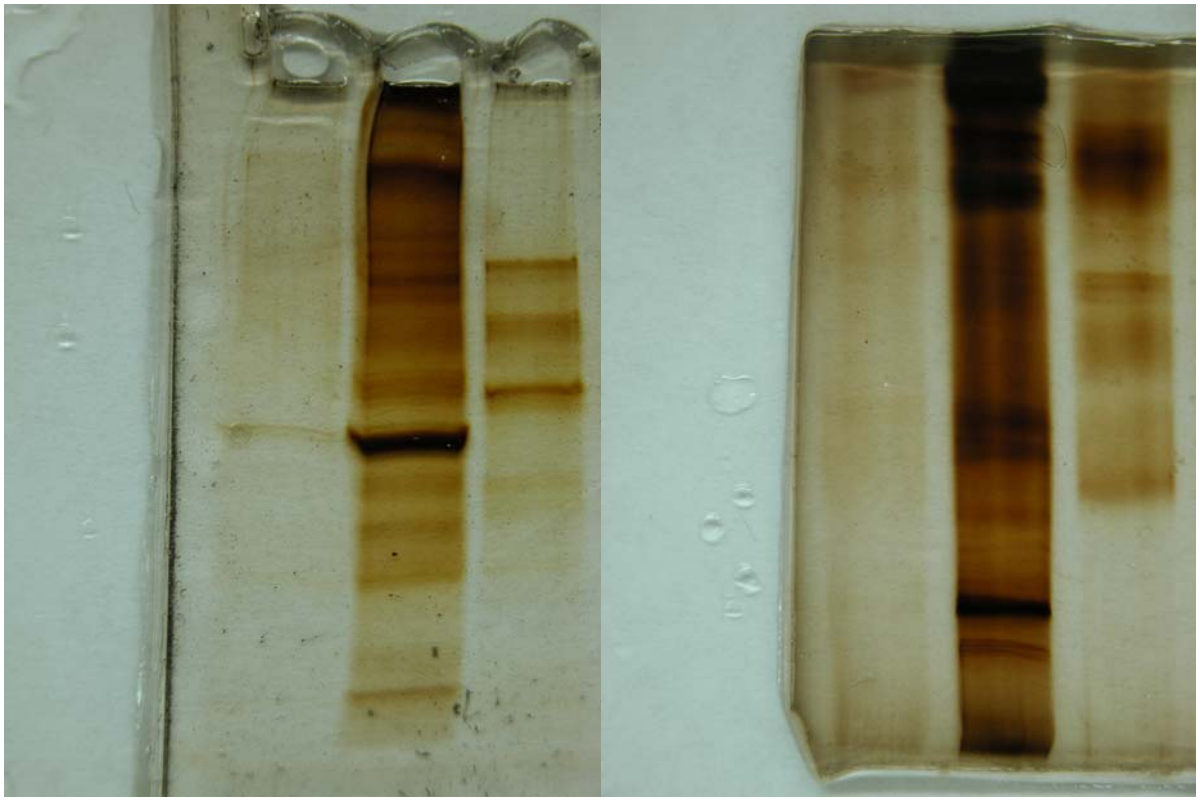

Figure 8. Two silver stained polyacrylamide denaturing gels, the left most is a 5%-20% gradient gel; the right is a standard 8% gel. Both gels contain the same samples. The left lane in each gel is FLAG and HA purified HeLa cell nuclear extract. The center lanes contains FLAG and HA purified nuclear lysate from HeLa cells over-expressing E2F8 DBD-1 mutant. The right most lanes contain FLAG and HA purified nuclear lysate from HeLa cells over-expressing E2F7 DBD-1 mutant.